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ORIGINAL RESEARCH ARTICLE

Cell-free DNA test for fetal chromosomal abnormalities in multiple pregnancies

Angel H. W. Kwan[1] | Maria Mar Gil[2,3] | Shuwen Xue[1] | Yvonne K. Y. Kwok[1] | Doris Lau[4] | Joanna Fung[1] | Andrea Chan[5] | Kwong Wai Choy[1] | Tak Yeung Leung[1] | Liona C. Poon[1]

[1]Department of Obstetrics and Gynecology, The Chinese University of Hong Kong, Hong Kong SAR

[2]Obstetrics and Gynecology Department, Hospital Universitario de Torrejón, Madrid, Spain

[3]Francisco de Vitoria University, Madrid, Spain

[4]Xcelom Limited, Hong Kong SAR

[5]Department of Obstetrics and Gynecology, United Christian Hospital, New Kowloon, Hong Kong SAR

Correspondence
Liona C. Poon, Department of Obstetrics and Gynaecology, Prince of Wales Hospital, The Chinese University of Hong Kong, Hong Kong SAR.
Email: liona.poon@cuhk.edu.hk

Abstract

Introduction: This study aimed to report the screening performance of cell-free DNA (cfDNA) testing for chromosomal abnormalities in twins, triplets, and vanishing twin pregnancies.

Material and Methods: Data were obtained from pregnant women with a multiple pregnancy or a vanishing twin pregnancy at ≥10 weeks' gestation who requested self-financed cfDNA testing between May 2015 and December 2021. Those that had positive screening results had diagnostic confirmatory procedures after counseling and consent. The performance of screening of the cfDNA test was determined by calculating confirmation rate and combined false-positive rate (cFPR).

Results: Data from 292 women were included after exclusion of those lost to follow-up, with no-result on cfDNA testing, or had reductions. Of the 292 pregnancies, 10 (3.4%) were triplets, including no cases of trisomy 21 and trisomy 18; 249 (85.3%) were twins, including 3 cases of trisomy 21 and no cases of trisomy 18 and 13; and 33 (11.3%) were vanishing twins, including 3 cases of trisomy 21 and 1 case of trisomy 18. The median (IQR) maternal age was 34 years (31–37). For triplet pregnancies, the initial no-result rate was 10.3% (95% confidence interval [CI] 3.6–26.4), all with results after redraw. For twin pregnancies, the initial no-result rate was 12.9% (95% CI 9.6–17.0), and the no-result rate after redraw was 1.6% (95% CI 0.7–3.6). For vanishing twins, there were no cases with no-result. All triplets had low-risk cfDNA results. The confirmation rate for trisomy 21 was 100% with a FPR at 0% due to the small number of positive cases for twins. For vanishing twins, one high-risk case for trisomy 21 and the only high-risk case for trisomy 18 were confirmed with a cFPR of 8.3% ($n = 2/24$; 95% CI 2.3–25.9).

Conclusions: cfDNA testing in twin pregnancies has sufficient screening performance for trisomy 21 but the number of affected cases for other conditions is limited to

Abbreviations: cfDNA, cell-free DNA; cFPR, combined false-positive rate; CI, confidence interval; CMA, chromosomal microarray analysis; IQR, interquartile range; QF-PCR, quantitative fluorescent polymerase chain reaction; SCA, sex chromosome aneuploidies.

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draw any meaningful conclusion. The use of cfDNA testing in triplet pregnancies and vanishing twins remains an area for further research.

KEYWORDS
cell-free DNA, Down syndrome, genome-wide, multiple pregnancies, screening for trisomies, screening performance, vanishing twin

1 | INTRODUCTION

Analysis of cell-free DNA (cfDNA) from maternal blood has been shown to be an effective method in the detection of a high proportion of fetuses affected by trisomies 21, 18, and 13.[1–10] For singleton pregnancies, the detection rate for the three major trisomies by the analysis of cfDNA in maternal blood is 99.7%, 97.8%, and 99.0%, for trisomies 21, 18, and 13, respectively, at a combined false-positive rate (cFPR) of 0.13%.[1]

Besides singleton pregnancies, the accuracy of cfDNA testing in multiple pregnancies has also been studied. cfDNA testing for twin pregnancies is feasible, however, the reported screening performance is lower.[11] The detection rates have been reported to be slightly lower at 99.0%, 92.8%, and 94.7% for trisomies 21, 18, and 13, respectively, but the number of reported cases is significantly fewer.[12] One of the possible reasons for lower detection rates is the lower fetal fraction that each twin contributes to the maternal circulation as compared to singletons.[11] Triplet pregnancies are rare, and as such, there is limited data on the use of cfDNA in such pregnancies.

Fetal cfDNA is mainly derived from the placenta and its small fragments are shorter than maternal cfDNA.[13,14] It has been found to be present in maternal blood from early pregnancy and is rapidly cleared after delivery.[15,16] However, when there is early fetal demise in one of the twins, the cfDNA fragments of the vanished twin have been demonstrated to persist in maternal blood for at least 15–16 weeks.[17,18] The number of trisomy cases analyzed in such circumstances is very small and therefore, it is not possible to accurately assess the performance of the cfDNA test for the screening of common trisomies in vanishing twins.

The objective of this study was to report the screening performance of cfDNA testing for chromosomal abnormalities in multiple pregnancies, including twins, triplets and vanishing twins.

Key message

cfDNA testing in twin pregnancies has sufficient screening performance for trisomy 21.

2 | MATERIAL AND METHODS

The data in this retrospective study were collected from consecutive pregnant women conceived by both natural conception and by assisted reproduction with a multiple pregnancy or a vanishing twin pregnancy at or beyond 10 weeks' gestation attending the Prince of Wales Hospital, Hong Kong SAR, who requested by their own request for and underwent self-financed cfDNA testing in screening for chromosomal abnormalities and variants (SafeT21 Express™, Xcelom, Hong Kong SAR) between May 2015 and December 2021. Ultrasound was performed to define the chorionicity by the T-sign for monochorionic twins and lambda sign for dichorionic twins.[19] Vanishing twin pregnancy was defined as a spontaneous loss of a fetus in a multigestation pregnancy either as an intrauterine sac with no fetal pole or a fetal pole with no fetal heart rate in early gestation, and with interval growth of the remaining fetuses.[20]

The cfDNA analysis was performed at our genome-wide cfDNA screening service provider laboratory. The study was approved by the Joint Chinese University of Hong Kong and New Territories East Cluster Clinical Research Ethics Committee on 30 October 2020 (CREC, Ref no. 2020:473). Prior to the test, the gestational age of the pregnancy was confirmed by the measurement of fetal crown-rump length of the larger twin or triplet and major fetal abnormalities were excluded by ultrasound scan.

Pre-test counseling was provided by obstetricians, written informed consent was obtained from all women for the cfDNA analysis. Aneuploidy calling for the cfDNA testing included trisomy 21, trisomy 13, trisomy 18, sex chromosome aneuploidies (SCAs) and seven microdeletion syndromes (22q11 deletion syndrome, 1p36 deletion syndrome, 2q33.1 deletion syndrome, Angelman syndrome, Cri-du-chat syndrome, Langer–Giedion syndrome, Prader–Willi syndrome). Regarding the screening performance in multiple pregnancies, in twin pregnancies, women were informed that the cfDNA test detects about 95% of fetuses with trisomy 21 in twin pregnancies, however the cfDNA test has uncertain screening performance for trisomies 18 and 13, SCAs and seven microdeletion syndromes.[21] For higher order pregnancies, the screening performance is also uncertain.

Cases which could hinder the interpretation of the results include trichorionic triamniotic triplets, vanishing twins, and those with fetal reduction. For these, detailed counseling regarding the uncertain screening performance with the chance of increased false-positive rates (FPRs) or need for unnecessary invasive testing was provided, and women consented to proceed with cfDNA testing after understanding the limitations. Women were

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informed that the results from the cfDNA test are available in 7–10 days.

Maternal blood was obtained by standard venepuncture (10 mL, in Streck cfDNA BCT™ tube, La Vista, Nebraska, USA) and sent via courier to the laboratory for cfDNA testing (SafeT21 Express™, Xcelom, Hong Kong SAR). cfDNA isolation, library construction, sequencing, and bioinformatics analyses were performed at the clinical laboratory of the genome-wide cfDNA screening service provider. Following library construction and amplification, the samples were sequenced on the Nextseq500 (Illumina, San Diego, CA) with a minimum of 20 million read pairs per sample. The fetal fraction level required for issuing a report was 8% for twins, 12% for triplets and 4% for vanishing twins with a suggested 8 weeks' interval from the fetal demise.[22]

Those that had positive screening results were offered diagnostic confirmatory procedures. All patients with positive genome-wide cfDNA screening results were offered follow-up prenatal diagnostic confirmatory procedures. Diagnostic laboratory tests included conventional cytogenetic studies, quantitative fluorescent polymerase chain reaction (QF-PCR), fluorescence in situ hybridization (FISH), karyotype and chromosomal microarray analysis (CMA) as deemed appropriate for the cases. DNA was extracted directly from uncultured samples (amniotic fluid, chorionic villi) for molecular investigations. For cases declining prenatal confirmatory testing, where possible we collected neonatal blood after birth for confirmation. The majority of diagnostic tests reported in this study were performed at the Prenatal Genetic Diagnosis Laboratory of the Department of Obstetrics and Gynaecology, The Chinese University of Hong Kong. QF-PCR utilizing short tandem repeat markers was performed on each prenatally acquired sample to detect maternal cell contamination and polyploidy. The CMA test utilized a well-established Fetal DNA Chip v2.0 (CGH + SNP, Agilent Technologies Inc., Santa Clara, CA, USA); this employs a custom panel for prenatal diagnosis targeted at the loci of 100 common microdeletion, microduplication conditions, and uniparental disomy of clinical relevance at high resolution, while providing whole genome coverage with a backbone resolution of 100 kb.[23]

2.1 | Pregnancy outcome

Results of the cfDNA testing were recorded in a secure database. Results from genetic testing, obtained from laboratories, and pregnancy outcome, obtained from obstetricians, general practitioners or the patients by a telephone interview at least 1 month after delivery, were recorded in the same database.

The outcomes were divided into firstly, trisomy 21, trisomy 18, trisomy 13, one of the SCAs, or one of the seven microdeletion syndromes if the QF-PCR, FISH, karyotype or CMA of chorionic villi, amniotic fluid or neonatal blood demonstrated the relevant chromosomal abnormality or genomic aberration; secondly, no trisomy 21, trisomy 18, trisomy 13, SCAs, or seven microdeletion syndromes if the QF-PCR, FISH, karyotype or CMA of chorionic villi, amniotic fluid or neonatal blood was normal, cases of high risk for SCA on the fDNA without a genetic confirmation were classified as sex discrepancies; thirdly, unknown QF-PCR, FISH, karyotype or CMA results because the pregnancies resulted in miscarriage or stillbirth and no diagnostic testing of fetal tissue was carried out; and fourthly, outcome unknown because the pregnancies were lost to follow-up.

2.2 | Statistical analyses

The results were divided into triplets (monochorionic, dichorionic, and trichorionic), twins (monochorionic, dichorionic), and vanishing twins.

Categorical variables were presented in counts and percentages, whereas median and interquartile range (IQR) were used for continuous variables. The performance of screening of the cfDNA test was determined by calculating confirmation rate (defined as the proportion of high-risk cfDNA results that were confirmed) and cFPR (defined as the proportion of high-risk results that did not correspond to an affected pregnancy) with 95% confidence interval (CI).

Data analyses were conducted with the statistical software package SPSS 22.0 (SPSS Inc., Chicago, IL, USA).

3 | RESULTS

3.1 | Study population

A total of 376 women with multiple pregnancies (29 triplets, 319 twins and 28 vanishing twins) requested for and underwent self-financed cfDNA testing but 52 cases were lost to follow-up and 5 had no-results on the cfDNA testing, and therefore not included in the final analysis (including 5 triplets, 44 twins and 8 vanishing twins). In addition, 27 multiple pregnancies that had spontaneous or iatrogenic reductions without genetic confirmation were also excluded.

Of the 292 included pregnancies, 10 (3.4%) were triplets, including no cases of trisomy 21 and trisomy 18; 249 (85.3%) were twins, including 3 cases of trisomy 21 and no cases of trisomy 18 and 13; and 33 (11.3%) were vanishing twins, including 1 cases of trisomy 21 and 1 case of trisomy 18. The median (IQR) maternal age was 34 years (31–37).

For the chorionicity in triplet pregnancies, none were monochorionic, 4 were dichorionic and 6 were trichorionic triplets. The median (IQR) fetal fraction was 16.6% (15.0–18.5) and 18.1% (12.8–18.7) for dichorionic and trichorionic triplets, respectively. In twin pregnancies, there were 84 and 165 monochorionic and dichorionic twins, respectively. The median (IQR) fetal fraction was 15.6% (11.8–18.4) for monochorionic and 13.7% (10.2–17.8) for dichorionic twin pregnancies. The median (IQR) fetal fraction for vanishing twins was 12.7% (9.9–15.6).

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3.2 | No result

There was a total of 44/376 (11.7%; 95% CI 8.8–15.4) cases with no-result after the first cfDNA blood sample, with the majority due to low fetal fraction (18, 40.9%). Other causes for no-result include interference and pre-lab errors. For triplet pregnancies, the initial no-results were 3/29 (10.3%, 95% CI: 3.6–26.4), all with results after redraw. For twin pregnancies, the initial no-results were 41/319 (12.9%, 95% CI: 9.6–17.0) and the no-results after redraw were 5/317 (1.6%, 95% CI: 0.7–3.6). This was due to persistent low fetal fraction, interference from fibroids and high body mass index. For vanishing twins, there were no cases with no-result. Of the 44 cases with no-result after first draw, 2 cases of twin pregnancies chose not to repeat the cfDNA test and opted for invasive testing.

3.3 | Triplet pregnancy

Pregnancy outcomes for the triplet pregnancies are documented in Table 1. All 10 pregnancies had low-risk cfDNA results which corresponded to their normal clinical pregnancy outcomes (Table 2).

3.4 | Twin pregnancy

There were 249 twin cases with outcomes as demonstrated in Table 3. For monochorionic twins ($n=84$), the confirmation rate for trisomy 21 was 100% ($n=1/1$; 95% CI 20.0–100.0) (Table 4). Additionally, one case demonstrated low level of chromosome Y on cfDNA, resulting in a pregnancy with babies with female genitalia; however, there was no confirmatory test performed prenatally or after delivery (Table 5).

For dichorionic twins ($n=165$), the confirmation rate was 100% for trisomy 21 ($n=2/2$; 95% CI 34.2–100.0) (Table 4). Additionally, there were 9 cases that were high risk for SCA (Table 5), including two cases with suspected monosomy X and 7 cases of low level of chromosome Y. For the two cases of suspected monosomy X, one case corresponded to two babies with male genitalia with no confirmatory test done prenatally and postnatally. The second case was likely due to contributions from the demised twin, with the live co-twin having amniocentesis showing 46,XX. All cases of low level of chromosome Y resulted in neonates with female genitalia.

Of these 9 cases of SCAs, three cases had diagnostic confirmatory testing by amniocentesis showing a normal karyotype. The remaining cases declined amniocentesis, and outcomes were based on clinical phenotype where the cases with low level of chromosome Y were of a female baby. All had an uncomplicated pregnancy course.

One case showed elevation of chromosome 8 on cfDNA with amniocentesis performed, demonstrating a duplication in 18q23 of 3 Mb, which was maternally inherited and resulted in an uncomplicated pregnancy and a phenotypically normal newborn.

3.5 | Vanishing twins

For cases of vanishing twins ($n=33$, Table 6), there were three cases that tested high risk for trisomy 21 with a confirmation rate of 33.3% ($n=1/3$; 95% CI 6.2–79.2). The remaining two high-risk cases for trisomy 21 had cfDNA testing done after the demise of the co-twin with amniocentesis performed in the live fetus showing a normal karyotype. There were two cases that were high risk for trisomy 18 along with ultrasound features suggestive of trisomy 18. One case was confirmed prenatally by chorionic villi sampling after ultrasound findings were suggestive of trisomy 18 (Table 7). The combined FPR was 8.3% ($n=2/24$; 95% CI 2.3–25.9).

One of the six cases that tested high risk for low level of chromosome Y declined amniocentesis (Table 8). The pregnancy was uncomplicated, and a baby with female genitalia was delivered at term. All other 5 had invasive testing with normal female karyotype.

4 | DISCUSSION

Our study demonstrated that cfDNA testing in twin pregnancies has sufficient screening performance for the trisomy 21. To date, available evidence for screening of multiple pregnancies by cfDNA was analyzed in a meta-analysis by Judah et al., in 2021, which included 13 studies. For trisomy 21, there were a total of 137 cases, and the pooled weighted detection rate and FPR for trisomy 21 were 99.0% (95% CI 92.0–99.9) and 0.02% (95% CI 0.001–0.43), respectively. For trisomy 18, there were 50 cases, and the pooled weighted detection rate and FPR were 92.8% (95% CI 77.6–98.0) and 0.01% (95% CI

TABLE 1 Pregnancy outcomes in triplet pregnancies.

Pregnancy outcome	Total	Fetal reduction	Final fetal number	cfDNA results
Dichorionic, $n=4$				
Live birth	4	No reduction	Triplet	Low risk
Trichorionic, $n=6$				
Live birth	5	No reduction	Triplet	Low risk
Miscarriage	1	Spontaneous	Miscarriage of whole pregnancy in 2nd trimester	Low risk

Abbreviation: cfDNA, cell-free DNA.

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TABLE 2 Screening performance in triplet pregnancies.

cfDNA results	Total	Pregnancy outcome	*n*	Confirmation rate (95% CI)	Combined FPR (95% CI)
Dichorionic, *n* = 4 (fetal fraction 16.6%, IQR 15.0, 18.5)					
Low risk	4	Normal	4	100% (0.51–1.0)	0%
Trichorionic triplets, *n* = 6 (fetal fraction 18.1%, IQR 12.8, 18.7)					
Low risk	6	Normal[a]	6	100% (0.6–1.0)	0%

Abbreviations: CI, confidence interval; FPR, false-positive rate.

[a]One pregnancy with second trimester miscarriage, karyotype was confirmed to be normal.

TABLE 3 Pregnancy outcomes in twin pregnancies.

Pregnancy outcome	Total	Fetal reduction	Final fetal number	cfDNA results	*N* (%)
Monochorionic, *n* = 84					
Live birth	80	No reduction	Twins	Low risk High risk for SCA - Low level ChrY	79 1
Miscarriage	2	Spontaneous	Miscarriage of whole pregnancy	Low risk	2
Termination of pregnancy	2	Spontaneous	Termination of whole pregnancy	Low risk - One twin IUD with co-twin fetal anemia High risk for trisomy21	1 1
Dichorionic, *n* = 165					
Live birth	161	No reduction	Twins	Low risk High risk for trisomy 21 High risk for SCA Increase in Chr8	150 1 8 1
		Spontaneous	Singleton	High risk for SCA	1
Miscarriage	2	Spontaneous	Miscarriage of whole pregnancy	Low risk	2
Termination of pregnancy	1	No reduction	Termination of whole pregnancy	Low risk (one twin with bilateral cleft lip and palate)	1
	1	No reduction	Termination of whole pregnancy	High risk for trisomy 21	1

Abbreviations: cfDNA, cell-free DNA; Chr, chromosome; IUD, intrauterine death; SCA, sex chromosomal abnormality.

TABLE 4 Screening performance of cfDNA testing for the three major aneuploidies in twin pregnancies, excluding high risk for other conditions.

cfDNA results	Total	Pregnancy outcome	*n*	Confirmation rate (95% CI)	Combined FPR (95% CI)
Monochorionic twins, *n* = 83 (fetal fraction 15.6%, IQR 11.8–18.4)					
Low risk	82	Normal	82	100% (96.0–100.0)	0%
High risk for trisomy 21	1	Trisomy 21	1	100% (20.0–100.0)	
Dichorionic twins, *n* = 155 (fetal fraction 13.7%, IQR 10.2–17.8)					
Low risk	153	Normal	153	100% (98.0–100.0)	0%
High risk for trisomy 21	2	Trisomy 21 - One twin in each case	2	100% (34.2–100.0)	

Abbreviations: cfDNA, cell-free DNA; CI, confidence interval; FPR, false-positive rate.

0.00–0.44). For trisomy 13, there were a total of 11 cases, and the pooled weighted detection rate and FPR for trisomy 13 were 94.7% (95% CI 9.14–99.97) and 0.10% (95% CI 0.03–0.13), respectively.[21]

Despite the small population in our study, the screening performance for trisomy 21 is comparable to that reported in the existing literature.

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TABLE 5 Screening performance of cfDNA testing for sex chromosome abnormalities in twin pregnancies and other rare autosomal trisomies.

cfDNA results	Total	Birth phenotype (sex)	*n*	Confirmation rate (95% CI)	Sex discrepancy (95% CI)
Monochorionic twins, *n* = 1 (fetal fraction 13.8%)					
High risk for any SCA - Low level of ChrY	1	Normal (female)	1	0%	100% (20.7–100)
Dichorionic twins, *n* = 10 (fetal fraction 13.4%, IQR 12.6–15.7)					
High risk for any SCA - Suspected monosomy X - Low level of ChrY	2 7	Normal[a] Normal (female)	9	0%	100% (72.2–100.0)
High risk for suspected Trisomy 8	1	Normal (male)	1	0%	

Abbreviations: cfDNA, cell-free DNA; Chr, chromosome; CI, confidence interval; FPR, false-positive rate; SCA, sex chromosomal abnormality.

[a]One case corresponded to two babies with male genitalia with no confirmatory tests, one case with amniocentesis showing 46,XX of the surviving co-twin after a demised twin.

TABLE 6 Pregnancy outcomes in vanishing twins.

Vanished twins, *n* = 33			
Characteristics	**Total**	**cfDNA results**	***N* (%)**
Live birth	31	Low risk	22
		High risk for SCA	6
		High risk for trisomy 21	2
		High risk for trisomy 18	1
Termination of pregnancy	2	High risk for trisomy 18	1
		High risk for trisomy 21	1

Abbreviations: cfDNA, cell-free DNA; SCA, sex chromosomal abnormality.

The accuracy of the cfDNA test is dependent on the proportion of fetal cfDNA in the maternal blood, and the minimum fetal fraction required is normally set at 4% for singleton pregnancies, with a lower fetal fraction leading to poorer accuracy.[5,24–27] The most common cause of no-result in both singleton and multiple pregnancy is a low fetal fraction,[28] which is also the reason for no-result in the majority of the cases for our study. The high no-result rate in multiple pregnancies is in contrast to the no-result rate of 2.08% in singleton from our lab.[22] Besides, the no-result cases had the cfDNA test performed between 12 and 18 weeks of gestation, not near the timing of fetal demise. It has been previously demonstrated that when the interval between blood draws is 14 days, there is an average increase in fetal fraction of 1.2%.[29] On this basis, a redraw was offered at least 3 weeks after the initial redraw as fetal fraction is positively correlated with gestational age.[30,31]

In a large series including 4615 twin pairs, it was demonstrated that the average fetal fraction for twin pregnancies was 32% higher than singleton pregnancies, however, individual contribution from each fetus in dizygotic twins was less than in singletons.[32] Struble et al. demonstrated that in monozygotic twins, the median total fetal fraction was 14%, whereas, in dizygotic twins, the median lowest fetal fraction contribution of the two fetuses was 8%.[33] For dizygotic twins, accurate cfDNA testing requires a minimum fetal fraction of at least 4% *per* twin, whereas for monozygotic twins, similar to singletons, the total fetal fraction for both twins together should be at least 4%.[33]

Our study showed a poor performance of the cfDNA test for the detection of SCA with 0% concordance for gender identification in cases of high risk of SCA in both twins and vanishing twins. Comparatively, Ting et al., demonstrated a positive predictive value of 40.0% (95% CI 13.7%–72.6%) for SCA in twin pregnancies.[34] As such, this low positive predictive value may lead to an unnecessary increase in invasive procedures and therefore, the use of cfDNA testing for screening of SCA in fetuses without ultrasound anomalies is still controversial.

Our results demonstrating that the cfDNA test has poor screening performance for vanishing twins are in contrast with the results of a large study conducted by Balaguer et al., which included 206 cases of vanishing twins and reported an initial FPR of 2.6%, that was reduced to 0.8% when cfDNA testing was performed after 14 weeks' gestation.[35] In our study, we had a cFPR of 8.3% (*n* = 2/24; 95% CI 2.3–25.9). The number of affected cases was too small in both studies to allow for any meaningful comparison. In the study conducted by Curnow et al., 42.1% of the 130 cases with false-positive results were due to the presence of vanishing twins.[36] The amount of cfDNA fetal fraction of the deceased fetus increases in maternal blood, peaking at up to 9 weeks after the demise, then decreases gradually over gestation to undetectable levels.[18] cfDNA

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TABLE 7 Screening performance of cfDNA testing for the three major aneuploidies in vanishing twins.

Total vanishing twins, $n = 26$[a] (fetal fraction 12.7%, IQR 9.9–15.6)					
cfDNA results	**Total**	**Pregnancyoutcome**	***n***	**Confirmation rate (95% CI)**	**Combined FPR (95% CI)**
Low risk	22	Normal	22	100% (85.3–100.0)	8.3% ($n = 2/24$) (2.3–25.9)
High risk for trisomy 21	3	Normal Trisomy 21	2 1	33.3% (6.2–79.2)	
High risk for trisomy 18	1	Trisomy 18	1	100% (20.7–100.0)	

Abbreviations: cfDNA, cell-free DNA; CI, confidence interval; FPR, false-positive rate.

[a]Not including one high risk for trisomy 18 without invasive test but with ultrasound features of trisomy 18.

TABLE 8 Screening performance of cfDNA testing for sex chromosome abnormalities in vanishing twins.

Total vanishing twins, $n = 6$ (fetal fraction 16.7%, IQR 12.3–24.9)					
cfDNA results	**Total**	**Birth phenotype(sex)**	***n***	**Confirmation rate (95% CI)**	**Sex discrepancy (95% CI)**
High risk for any SCA - Low level of ChrY	6	Normal (female)	6	0% (0–39.0)	100% (61.0–100.0)

Abbreviations: cfDNA, cell-free DNA; Chr, chromosome; CI, confidence interval; FPR, false-positive rate; SCA, sex chromosomal abnormality.

analysis can detect the presence of DNA material from the demised twin up to 15–16 weeks after the demise, therefore it has been proposed to delay the testing until the second trimester.[17,18,35] A more recent approach using a single-nucleotide polymorphism-based method has successfully identified cfDNA derived from the vanishing twin in the maternal circulation, but it has not been proven that this help provide accurate results for the live fetus.[36] However, there is still a lack of large systematic studies to fully analyze the persistence of cfDNA from a vanishing twin in the maternal circulation and therefore, we cannot provide accurate counseling to prospective parents in terms of performance or no-result rate.

One of the main strengths of our study was that we were able to obtain pregnancy outcome data in up to 85% of cases. Maternal and fetal outcomes were documented to allow for correlation with the cfDNA results. There is limited evidence for the use of cfDNA screening in vanishing twins and triplet pregnancies, therefore, the screening performance is unknown. However, in our cohort, women with these types of pregnancy self-requested and provided informed consent for cfDNA testing, after adequate counseling and fully understanding the limitations. This allowed us to add a small series of vanishing twins and triplet pregnancies to the existing literature.

There were several limitations in our study. First, the number of cases affected by any trisomy in twin pregnancies was very small for accurate estimation of the test performance. Besides, we were unable to report on the screening performance in triplets as there were no confirmed aneuploidy cases. Second, we were unable to obtain post-delivery genetic confirmation for the cases of vanishing twin to correlate with a positive cfDNA result, so we were not able to investigate the reasons for the discordant results further.

5 | CONCLUSION

cfDNA testing in twin pregnancies shows a sufficient screening performance for trisomy 21 but the number of affected cases for other conditions is limited to draw any meaningful conclusion. In addition, the use of cfDNA testing in triplet pregnancies and vanishing twins remains an area for exploration and further research is needed before its introduction into routine clinical practice.

AUTHOR CONTRIBUTIONS

Angel H.W. Kwan: conceptualization, formal analysis, investigation, writing, original draft, writing, review and editing. Maria Mar Gil: conceptualization, formal analysis, writing, review and editing. Shuwen Xue: investigation. Yvonne K.Y. Kwok: Data curation, methodology. Doris Lau: Data curation. Joanna Fung: investigation. Andrea Chan: Investigation. Kwong Wai Choy: data curation, methodology. Tak Yeung Leung: Data curation, methodology. Liona C Poon: conceptualization, formal analysis, methodology, writing, review and editing, supervision.

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CONFLICT OF INTEREST STATEMENT

Doris Lau is an employee of Xcelom Limited, Hong Kong.

ETHICS STATEMENT

The study was approved by the Joint Chinese University of Hong Kong and New Territories East Cluster Clinical Research Ethics Committee on October 30, 2020 (CREC, Ref no: 2020:473).

ORCID

Angel H. W. Kwan https://orcid.org/0000-0002-4140-6651
Maria Mar Gil https://orcid.org/0000-0002-9993-5249
Kwong Wai Choy https://orcid.org/0000-0002-3616-6200
Tak Yeung Leung https://orcid.org/0000-0002-9153-0343

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